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INST ZELLBIOLOGIE

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S. 03

In the United States Patent Office

In re Application of

Juergen Bode, Jost Seibler and Dirk Schuebeler

Serial-No. 09/841,843

Filed: April 25, 2001

Title: Method for marker-free repetitive DNA expression
of cassette exchange in the genome of cells or
parts of cells

DECLARATION

1. I, Alfred Nordheim, residing in Auf der Morgenstelle 15,
72076 Tuebingen, Germany, am a citizen of the Federal
Republic of Germany. I am Professor for Molecular Biology
at the Institute for Cell Biology at the University of
Tuebingen. My curriculum vitae is enclosed. I am trained
and skilled in the field of stem cell research, in
particular embryonic stem cells (ES cells). I have done
extensive research in the field of homologous and site-
specific recombination experiments in various cell types.
A publication list is enclosed.

I have carefully read and fully understood the US patent
application of Juergen Bode, Jost Seibler and Dirk
Schuebeler, US Serial No. 09/841,843 filed on April 25,
2001. Additionally, I have studied and fully understood
the prior art references

i) Thomas Schlake and Juergen Bode, 1994 Biochemistry,
33:12746-12751 "Use of Mutated FLP Recognition Target

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(FRT) Sites for the Exchange of Expression Cassettes
at Defined Chromosomal Loci";

- ii) Ludwig et al., 1996, Transgenic Research 5:385-395
"FLP-mediated site-specific recombination in
microinjected murine zygotes"; and
- iii) Jung et al., 1993, Science 259: 984-987 "Shutdown of
Class Switch Recombination by Deletion of a Switch
Region Control Element"

3. The invention described in the US patent application
09/841,843 concerns a method for the recombinase mediated
cassette exchange (RMCE), which allows the exchange of a
first DNA cassette against a second incoming DNA cassette.
The exchange is mediated by FLP recombinase in conjunction
with sets of heterospecific FLP recombinase target (FRT)
sequences (page 4, third paragraph of the application).

The application uses embryonic stem cells (ES cells) (page
5, second paragraph and page 8, first paragraph of the
application).

For RMCE, the application uses a positive/negative
selection strategy based on neomycin for positive
selection and ganciclovir (GANC) for negative selection.
For tagging the chromosomal loci, the target construct
 $F_3hygtkF$ (positive selection for hygromycin resistance)
and F_3neoF or $F_3P^{PGK}neoF$ (for negative selection with
ganciclovir) were used (page 8, third paragraph and page
9, second paragraph of the application).

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In order to reduce the accumulation of inactivated clones prior to negative selection, the application features the combined positive and negative selection, and the positive selection is maintained during cultivation prior to the negative selection (page 13, paragraph following table 2 of the application). The application reports a targeting efficiency for positive plus negative double selection in the extraordinary high range of 54 and 100 % (page 15, first paragraph and table 3 of the application).

4. The prior art reference (i) Schlake and Bode 1994 Biochemistry 33:12746-12751 deals with an FLP/FRT system for site-specific recombination in cultured mammalian cells. Schlake and Bode were aware of the problems connected with homologous recombination occurring in embryonic stem cells and therefore chose to use established cultured mammalian cells (BHK or CV-1 cells) because they have lost the potential to perform homologous recombination. According to the understanding of Schlake and Bode especially BHK cells have a long track record for the safe production of vaccines (Schlake and Bode, page 12746, left column, first paragraph).

Schlake and Bode used hygromycin B and ganciclovir for selection (page 12747, left column, fourth paragraph), however, selection conditions are not described as set to maintain the positive selection by hygromycin all the time until the exchange of the first DNA expression cassette against the second incoming DNA expression cassette is completed.

Schlake and Bode use the plasmids F5HygTkF and F5NeoF for their experiments (page 12746, right column, second but last paragraph and page 12747, figure 1).

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Schlake and Bode tested different plasmids with respect to their recombination efficiency and received enormous differences when different spacers (F_n , n being 0, 1, 2, 3, 4 or 5, page 12748, right column, table 1). Strikingly, the use of F_3 spacer led to an efficiency of only 0,24 (0,51) %, is a conf interval of only 1,5 %. In accordance with these experimental results, Schlake and Bode observed an increasing discrimination of the mutated spacer in the order of $F_1 < F_4 = F_2 < F_3 < F_5$ (page 12749, right column, first paragraph) and Schlake and Bode point to the fact that these observations leave unresolved the question as to the extend of $F_n \times F_n$ self-recognition. They further point out, that the signal strength in another experiment decreases in the order of $F >> F_1 > F_4 > F_2 > F_3 > F_5$, which reproduces the conclusions from table 1 (page 12750, left column, first paragraph). From these experiments it can be gained that F_3 as the omitted spacer which was used in the application does not seemed be of any particular advantage. The overall efficiency of the experiments reported by Schlake and Bode with omitted spacer, were 1,7 - 0,15 % (page 12749, paragraph bridging left and right column), which is significantly less to the reported efficiencies of the application of 54 and 100 % respectively (page 15, table 3).

5. When comparing the teaching of Schlake and Bode and the present application, significant differences in the disclosure are apparent. These are summarized in the following table.

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	Invention US application 09/841,843	Schlake and Bode Biochemistry 1994 33:12746 - 12751
Cells	Embryonic stem cells	BHK and CV-1 cells. embryonic stem cells are excluded because cells with no potential for homologous recombination should be used (page 12746, left column, first paragraph)
Hygromycin and neomycin constructs	F ₃ hygtkF and F ₃ NeoF (page 8, third paragraph and page 9, second paragraph)	F5HygtkF and F5NeoF (page 12747, figure 1)
Selection conditions	Maintenance of conditions for positive selection until the integration of the second DNA expression cassette is completed (claim 1 of the application)	
Efficiency	Targeting frequency F18, F21 and F22 is 100 %, 54 %, 100 % respectively (table 3, page 15)	Targeting frequency for F ₃ is 0,24, 0,51 and 1,5 % respectively (table 1, page 12748)

For a person skilled in the art the teaching of the present invention is entirely surprising since the prior art teaches very low recombination efficiencies while the application achieves very high efficiencies at three different loci and is therefore a significant improvement over the prior art.

6. The prior art reference of Ludwig et al. 1996, Transgenic Research 5:385-395 reports that introduction of a FLP-

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recombinase expression vector into transgenic one-cell fertilized mouse eggs induces a recombination event at a chromosomal FRT target locus (see abstract on page 385). Ludwig et al. discloses a classical FLP-mediated site-specific recombination between tandem arrays of FRT sites (page 393, figure 6). The frequency of recombination is not explicitly given, but it is merely referred to as "detectable" (page 393, left column, last line). The reference of Ludwig et al. therefore falls into the category of the prior art references mentioned in the application on page 3, third paragraph, as it also leaves behind a selectable marker and potentially unwanted vector sequences which are also inserted. Therefore, even though Ludwig et al. use mouse embryos, it is a prior art reference with the draw backs mentioned in the introduction of the application.

7. The reference of Jung et al. 1993, Science 259:984-987 refers to the class switching of immunoglobulins mediated by a homologous recombination event. It allows B cells to sequentially express antibodies that have identical specificities but that differ in class and thus effector function (page 984, left column, first few lines and right column, second paragraph). This study uses murine embryonic stem cells for the homologous recombination event. Consecutively the new cassette was deleted by the FRT recombination system, which is a site-specific deletion. As a result, the targeted locus retains a single copy of a FRT signal (page 965, left column, first paragraph).

Therefore, even though Jung et al. use embryonic stem cells, the system for recombination are the classical homologous recombination and the site-specific recombination which are described in detail on pages 1-3

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of the present application. In particular, it is explicitly mentioned in Jung et al. that vector sequences are left behind in the genome, which is one of the goals. the present invention circumvents.

8. Combining the teachings of Schlake and Bode with either Ludwig et al. or Jung et al., the person skilled in the art would not reach the claimed invention. The prior art references Ludwig et al. and Jung et al. use embryonic stem cells and murine embryos, respectively, the technology disclosed in these references is either classical site-specific recombination and/or homologous recombination, which all comprise major draw backs discussed in the invention. Therefore, a person skilled in the art, when starting from the disclosure of Schlake and Bode would not gain any further information from Jung et al. or Ludwig et al. in order to reach the claimed invention. In particular, a person skilled in the art would not be able to solve the problem of integrated vector sequences or low efficiencies in recombination together with the requirement for an incoming selectable marker.

Therefore, it is believed that the teaching of the present invention is not obvious from the prior art references.

9. I further declare, that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like, so made, are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the US Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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Tübingen

Jan. 20, 2004

A. Nordheim

Date and Signature

Enclosures:

Curriculum Vitae

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96 Heidenreich, O., Neininger, A., Schratt, G., Zinck, R., Cahill, M. A., Engel, K., Kotlyarov, A., Kraft, R., Kostka, S., Gaestel, M., and Nordheim, A. (1999). MAPKAP kinase 2 phosphorylates serum response factor in vitro and in vivo. *J Biol Chem* 274, 14434-14443.

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Prof. Dr. Alfred Nordheim - Patents

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	1997	Full Professor of Molecular Biology, Tübingen University, Germany
Research and Teaching Experience:	1980-81	Postgraduate scientist, MIT, Cambridge, USA
	1982-83	Research Associate, MIT, Cambridge, USA
	1984-89	Head of research group, ZMBH, University of Heidelberg, Germany
	2000	Guest Professor (6 months), MIT, Whitehead Institute, Cambridge USA. Host: Prof. Dr. R. A. Weinberg
Scientific Administration:	1989-1996	Director of the Institute for Molecular Biology at Hannover Medical School, Germany
	1989-	"Speaker" for the DFG-Graduate College "Molecular Pathophysiology of Cell Growth"
	1992-	Deputy Speaker for the SFB 265 "Immune Reactions and Pathomechanisms during Organ Transplantation", Hannover, Germany
	1996-	Coordinator of the IEC Network "Signalling Networks in Development and Disease" (TMR Network)
	1997-	Director of the Department of Molecular Biology, Interdisciplinary Institute for Cell Biology, Eberhard-Karls-University of Tübingen,
Scientific Awards and Functions:	1991	Elected member of the "European Molecular Biology Organization (EMBO)"
	1992	Max-Planck-Research Prize of the Max-Planck Society and the Alexander von Humboldt Foundation (together with R.A. Weinberg, MIT, USA)
	1997-	Member of the Research Advisory Committee of the Medical Faculty at Eberhard-Karls- University of Tübingen
	1998-2001	Member of the Expert Advisory Board at the Max-Planck-Institute for Developmental Biology, Tübingen
	1999-	Member of the Rector's Committee for Research "Questions" at Eberhard-Karls- University of Tübingen

	2002-	Member of the Scientific Advisory Board of the Dr. Mildred Scheel Foundation for Cancer Research, Bonn, Germany
Duties as Scientific Reviewer:	2002-	Vice-President of the German Genetics Society (GfG)
	1986-	Reviewer for various national and international scientific organizations (Switzerland, Great Britain, USA, Germany) and for different scientific journals (Cell, EMBO Journal, Nature, Molecular & Cellular Biology, J. Mol. Biol., Nucleic Acids Research, etc.)
	1988-	Reviewer for grant applications, Schwerpunktprogramme and various SFB programs of the German Research Society (DFG)
	1992-	Austrian Funds for the Advancement of Scientific Research (FWF): Reviewer for various grant programs and the SFB 002 in Innsbruck (Austria)
	1992-1998	Appointment as scientific advisor to the German-Israeli Foundation for Scientific Research and Development (GIF)
	1995-	Reviewer for the German National Board of Academic Advisors ("Wissenschaftsrat") (Köln)
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Membership to scientific organizations:

- Society for Biological Chemistry (FEBS)
- Society for Genetic Engineering
- European Molecular Biology Organization (EMBO)
- American Association for the Advancement of Sciences (AAAS)
- "Förderverein Molekularbiologie und Biomedizin" (Founding member, 1st president)
- New York Academy of Sciences
- German Association for Gene Therapy
- Deutsche Krebsgesellschaft e.V.
- German Society for Proteome Research

Founding of a company:

Feb. 2000	Founding of the company "ProteoMed GmbH", Tübingen; Position as CEO
Feb. 2001	Merger of "ProteoMed GmbH" with "ProteoSys GmbH" to form "ProteoSys AG", Mainz, Position as Scientific Advisor.